Docket No. 56972/JPW/AKC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Honorable Assistant Commissioner for Patents
Washington, D.C. 20231

July 28, 1999

S I R:

Transmitted herewith for filing are the specification and claims of the patent application of:

Chong-Jin Oon, Wei-Ning Chen, Ai-Lin Leong, and Koh Shiuan for
Inventor(s)

DETECTION OF HUMAN HEPATITIS B VIRUS SURFACE ANTIGEN MUTANTS BY SPECIFIC AMPLIFICATION AND ITS APPLICATION ON GENE CHIP
Title of Invention

Also enclosed are:

X 1 sheet(s) of ____informal X formal drawings.

____ Oath or declaration of Applicant(s).

____ A power of attorney

____ An assignment of the invention to _____

____ A Preliminary Amendment

____ A verified statement to establish small entity status under 37 C.F.R. §1.9 and §1.27.

The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT

	NUMBER FILED		NUMBER EXTRA*		RATE SMALL ENTITY	RATE OTHER ENTITY		FEE SMALL ENTITY	FEE OTHER ENTITY
Total Claims	23 -20	=	3	X	9	18	=	$	$ 54
Independent Claims	5 -3	=	2	X	39	78	=	$	$156
Multiple Dependent Claims Presented:	X Yes ____No				130	260	=	$	$260
*If the different in Col. 1 is less than zero, enter "0" in Col. 2					BASIC FEE			380	760
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__X__ Filing fees under 37 C.F.R. §1.16.

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__X__ Three copies of this sheet are enclosed.

_____ A certified copy of previously filed foreign application No. _____ _____ filed in _____ on _____. Applicant(s) hereby claim priority based upon this aforementioned foreign application under 35 U.S.C. §119.

__X__ Other (identify) Claims 6 Pages; Abstract 1 Page; One loose set of formal drawing; Express Mail Certificate of Mailing bearing Label No. EL278886760US, dated July 28, 1999.

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Application
for
United States Letters Patent

To all whom it may concern:

Be it known that **Chong-Jin Oon, Wei-Ning Chen, Ai-Lin Leong, and Koh Shiuan**

have invented certain new and useful improvements in

DETECTION OF HUMAN HEPATITIS B VIRUS SURFACE ANTIGEN MUTANTS BY SPECIFIC AMPLIFICATION AND ITS APPLICATION ON GENE CHIP

of which the following is a full, clear and exact description.

DETECTION OF HUMAN HEPATITIS B VIRUS SURFACE ANTIGEN MUTANTS BY SPECIFIC AMPLIFICATION AND ITS APPLICATION ON GENE CHIP

Technical Field

This invention relates to nucleic acid amplification with subsequent hybridization on solid supports (gene chip on glass support) as its application. More specifically, it relates to novel nucleic acid probes for detecting human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) in serum samples.

Throughout this invention, various publications are referenced by Arabic numerals. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

Background of the Invention

The present invention concerns the specific detection from serum samples of human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine), by polymerase chain reaction, using novel nucleic acid probes. The present invention could be applied to detection of such mutant by other means, in particular detection of differential fluorescent signals after hybridization of unknown human viral DNA samples with a specific nucleic acid probe that is immobilized on solid supports (i.e. glass).

Viral hepatitis is a systemic disease involving primarily the liver, with hepatitis B virus being mainly responsible for most cases of acute or chronic hepatitis.

Antigenic characterization of human hepatitis B virus derives from the complex protein found on the virus' surface, namely hepatitis B virus surface antigen. The major antigenic epitope, designated as '**a**' and located

from amino acid 124 to 147 of the hepatitis B virus surface antigen, is common to all hepatitis B virus. This 'a' epitope is directly involved in inducing neutralizing antibodies against hepatitis B viral infection. Such induction can be achieved by immunizing individuals with commercial available vaccines, consisting of non-infectious subviral hepatitis B surface antigen particles. An acquired protection in humans against hepatitis B viral infection is generally indicated by the presence of an adequate amount of serum antibody to hepatitis B virus surface antigen (anti-HBs). There is also a concomitant decrease of the serum viral surface antigen. However, an increasing numbers of incidence of hepatitis B viral infection despite the serum anti-HBs have been reported. These are largely contributed by hepatitis B viral strains that carry mutations on the antigenic region of the viral surface antigen, and in particular on the 'a' epitope. Such surface antigen mutants are of serious concern as they display reduced affinity for the neutralizing antibodies and able to replicate independently. The most common mutation among these vaccine-escape hepatitis B virus variants has been found at amino acid residue 145 (Glycine to Arginine) on the 'a' epitope of the viral surface antigen. In immunized infants born to HBeAg positive mothers, for example, the mutation 145 (Glycine to Arginine) within the major hydrophilic region is the most common variant found in those who subsequently become infected despite adequate amount of protective anti-HBs antibodies. This particular mutant is also the most common variant found in orthotopic liver transplantation patients who succumbed to hepatitis B viral infection despite immunoprophylaxis using hepatitis B immunoglobulin. Significantly, this human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) is also a naturally occurring variant that has been detected worldwide. In Singapore, despite the fact

that an active vaccination program has resulted in a significant decrease of acute hepatitis B infection and the incidence of primary hepatocellular carcinoma in the general population, cases of breakthrough viral infection have been detected. Many of them (twelve out of forty-one) carry the viral surface antigen mutation 145 (Glycine to Arginine).

The emergence of this replicative hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) and its ability to escape detection using currently available reagents are of grave concern, because this mutant is infectious and has resulted in the development of acute hepatitis B in Europe as well as Singapore. Our latest data also point to an increasing incidence of quasispecies in Singapore population consisting of both wild type and surface antigen 145 (Glycine to Arginine) of hepatitis B virus. Although serum human hepatitis B viral DNA can be detected by standard liquid hybridization assay (Abbott Laboratories, U.S.A.), such commercial kits are not designed to distinguish wild type hepatitis B virus from variants carrying mutations on hepatitis B surface antigen. A rapid and simple detection method for this particular human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) would therefore be useful for its diagnosis, therapy and prevention.

One approach toward this goal would be to detect the specific nucleic acid sequence of the HBsAg mutant 145 (Glycine to Arginine) in serum samples by specific Polymerase Chain Reaction amplification. Specific oligonucleotides would need to be designed on the basis of various available HBsAg sequences. Current methods of nucleic acid and oligonucleotide identifications have problems of sensitivity and selectivity, and have disadvantages such as the tedious and cumbersome

analysis of the amplification results requiring highly skill operators to carry out the analyses *i.e.* agarose gel, polyacrylamide gel and molecular cloning. Application by the sophisticated oligonucleotide-based chip (Gene Chip) technology can provide further improved accuracy and rapid diagnostic screening assay. Gene Chip technology is now making more efficient and easier to use tools possible for obtaining and evaluating genetic information. This technology can be used for a broad spectrum of applications and analysis, such as sequence analysis, genotyping and monitoring of gene expression.

First developed in the late 1980s as a concept to determining DNA sequence by hybridization, the Gene Chip technology has been used in various fields of medicine and pharmaceutical research. Usually immobilized on solid support such as glass, the probe sequences can be originated from different procedures. These include the photolithographic synthesis of 20-25-mer oligonucleotides onto silicon wafers (Affymetrix, Glaxo-Welcome), printing of 500-5000 nucleotide cDNAs onto glass chip or dotting of pre-synthesized specific oligonucleotides (via their chemically modified terminus) onto glass chip. For the purpose of detecting hepatitis B surface antigen mutants with high specificity, the limited number of possible mutation sites (amino acid 100-160) would favor the dotting of pre-made oligonucleotides onto glass support in the application of Gene Chip technology. A specific detection system for hepatitis B surface antigen Glycine-145-Arginine mutant has been developed and described in this disclosure. It is based on novel nucleic acid probes which constitute an important innovative step towards the goals mentioned. Their identification would contribute to the effective prevention and control of hepatitis B viral infection

arising from these viral surface antigen mutants, through rapid screening in blood banks, commercial and research diagnostic laboratories. This know-how information can also be used to detect other hepatitis B mutants.

The specific detection system described in the present invention that is based on novel nucleic acid probes constitutes an important step towards these goals and should contribute to the effective control of hepatitis B viral infection arising from these viral surface antigen mutants.

Summary of the Invention

This invention describes novel nucleic acid probes that can be used in polymerase chain reaction to amplify specifically human hepatitis B surface antigen mutant 145 (Glycine to Arginine) from serum samples. In contrast to commercial liquid hybridization assays, the detection system in the present invention is able to detect the said human hepatitis B viral mutant from serum samples with specificity. The specificity of the present invention in turn allows detection of such mutant using gene chip technology, wherein the specific fluorescent labeled nucleic acid probe is immobilized on solid glass supports prior to hybridization reaction with target viral DNA fragment labeled by different fluorescent dye, and amplified from serum samples. The specific detection of this infectious human vaccine-escape hepatitis B surface antigen mutant 145 (Glycine to Arginine), from quasispecies serum samples, provides useful information for future monitoring of such mutants using specific therapeutic vaccines and effective antiviral agents.

Brief Description of the Figure

Figure 1 is a photograph that shows electrophoresis pattern of the polymerase chain reaction product using either plasmid or viral DNA as template. The plasmid is pcDNA3.1 (InvitroGen, U.S.A.) containing the coding region of the human hepatitis B virus surface antigen, either the wild type (*lane 1*) or the mutant 145 (Glycine to Arginine) (*lane 2*). The viral DNA is isolated from serum samples containing either the wild type (*lane 3*) or mutant 145 (Glycine to Arginine) (*lane 4*) human hepatitis B virus. Human hepatitis B virus DNA is extracted from serum sample by phenol/chloroform. Polymerase chain reaction amplification is carried out in the present invention using specific oligonucleotides.

Detailed Description of the Invention

This invention provides a method for detecting specifically from serum samples the human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine), by polymerase chain reaction. Direct application of this detection system includes the detection of human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) by detecting differential fluorescent signals, following hybridization of human hepatitis viral DNA with novel nucleic acid probes immobilized on solid glass supports.

The subject matter of the present invention is the development of a simple, sensitive method to detect human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) from serum samples. As a direct application, detection of this particular mutant could be achieved by detecting differential fluorescent signals after hybridization of the novel nucleic acid probes immobilized on solid glass supports and target viral DNA sequences.

The present invention consists of the design of novel oligonucleotides that can be used in polymerase chain reaction, to specifically amplify the human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine). A set of synthetic oligonucleotides useful as amplifier probes, comprise at least two different oligonucleotides probes, wherein each oligonucleotide probe consisting of:

1. first oligonucleotide having 14 nucleotides (5'-TACGGACAGAAACT-3', position 582 to 595 as referred to the wild type human hepatitis B virus genome) which contains the mutation G to A, leading to change at amino acid 145 of hepatitis B virus surface antigen (Glycine to Arginine), at position

8 of the oligonucleotide;

2. second oligonucleotide having 21 nucleotides (5'-TTAGGGTTTAAATCTATACCC-3', position 842 to 822 as referred to the wild type human hepatitis B virus genome), which is complementary to the coding strand of hepatitis B virus surface antigen

Further subject matters of the present invention include reagents for implementing the method, namely the viral DNA extraction from human serum samples, the polymerase chain reaction.

One of the applications of the present invention is the detection of the human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) using a solid glass supports device. In the present invention, further modifications have been added to two oligonucleotides (listed in Claim 5): 5'-TACGGACGGAAACT-3', and 5'-TACGGACAGAAACT-3', both located from position 582 to 595 as referred to the wild type human hepatitis B virus genome. These modifications include a fluorescent dye, 6-(fluorescein-6-carboxamido) hexanoate (6FAM), at its 5' terminus and a primary amine group at its 3' terminus. The resulting oligonucleotides that are immobilized on solid glass supports have the following structure: 5'-(6FAM)TACGGACGGAAACTGTTTTTTTTTTTT (C-7 amine)-3', and 5'-(6FAM)TACGGACAGAAACTGTTTTTTTTTTTT (C-7 amine)-3', and the second oligonucleotide contains the mutation G to A (position 8) leading to change at amino acid 145 (Glycine to Arginine) of human hepatitis B virus surface antigen. There is also an inclusion of a poly-T (underlined) as a synthetic linker aiming at facilitating the subsequent hybridization reaction with target human viral DNA sequences from serum samples.

The present invention also concerns the design of

synthetic oligonucleotides that can be used to amplify target human hepatitis B virus surface antigen DNA from serum samples, prior to their hybridization with the oligonucleotide immobilized on solid glass supports as mentioned above. Specifically, these oligonucleotides used in polymerase chain reaction to generate amplified product of less than 150 base pairs and consist of the following structure:

1. first oligonucleotide having 20 nucleotides with a biotin group at its 5' terminus (5'-Biotin-AGGATCAACAACAACCAGTA-3', and located from position 489 to 508 as referred to the wild type human hepatitis B virus genome). The presence of a biotin group allows the separation of amplified DNA fragments using streptavidin magnetic particles;
2. second oligonucleotide having 20 nucleotides with a fluorescent dye Texas Red at its 5' terminus, and complementary to the coding strand of human hepatitis B virus surface antigen (5'-Texas red-ATCGTCCTGGGCTTTCGCAA-3', and located from position 634 to 615 as referred to the wild type human hepatitis B virus genome).

In accordance with the present invention, serum samples may contain the human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine). The present invention enables its specific detection. For detection by means other than visualizing human hepatitis B viral surface antigen mutant 145 (Glycine to Arginine) DNA fragments, the present invention can be further developed into gene chip whereby the oligonucleotide used in specific amplification of the said hepatitis B viral mutant is immobilized onto solid glass supports. The presence of the human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) in a particular serum samples can

be detected by fluorescence signals upon hybridization with the immobilized oligonucleotide.

Examples of applications of the present invention are shown below, however, the present invention shall in no way be limited to these examples.

EXAMPLES

General Experimental Procedures

Viral DNA from serum carrying either the wild type or mutant 145 (Glycine to Arginine) of the human hepatitis B virus surface antigen is isolated as follows. 200 μl of the serum sample was added to 400 μl of lysis buffer (Tris chloride 10 mM, pH7.4, EDTA 1 mM, and sodium dodecyl sulfate 2%) and 25 μl of proteinase K (20 mg/ml), incubated at 65°C for 3 hours. Viral DNA is then extracted by phenol/chloroform and precipitated by ethanol.

The coding region of the human hepatitis B virus surface antigen, either wild type or mutant 145 (Glycine to Arginine), is amplified by polymerase chain reaction using the following oligonucleotides:

1. The 5' oligonucleotide is a sense oligonucleotide that matches the start site of the human hepatitis B surface antigen (5'-ATGAATTCATGGAGAGCACAACATCAGGATTCCTA-3' and located from position 157 to 183 as referred to the wild type human hepatitis B viral genome), wherein the underlined nucleotides represent an additional site for restriction enzyme EcoRI;
2. The 3' oligonucleotide is an anti-sense oligonucleotide that matches the stop site of the human hepatitis B surface antigen

(5'-GAGAATTCTCAAATGTATACCCAAAGACAAAAGAA-3', located from position 811 to 837 as referred to the wild type human hepatitis B viral genome), wherein the underlined nucleotides represent an additional site for restriction enzyme EcoRI;

Polymerase chain reaction using viral DNA as template is then carried out on a DNA Thermal Cycler (Perkin-Elmer, Cetus) for 35 cycles using *Pfu* polymerase (Stratagene, U.S.A.). Cycling conditions consist of 1.5 minutes at denaturing temperature (94 °C), 2 minutes at annealing temperature (53 °C) and 2 minutes at extension temperature (72 °C).

Amplified viral DNA fragment (human hepatitis B virus surface antigen, either wild type or mutant 145 (Glycine to Arginine)) is subjected to restriction enzyme by EcoRI, prior to cloning into pcDNA3.1 plasmid (InvitroGene, U.S.A.) pretreated by the same restriction enzyme.

For the novel detection system in the present invention, polymerase chain reaction is carried out using either plasmid DNA (containing coding region of either wild type or mutant 145 (Glycine to Arginine) of human hepatitis B virus surface antigen), or viral DNA as indicated in Figure 1. Oligonucleotides used in the said polymerase chain reaction are listed in Claim 1 and have the following localization on the wild type human hepatitis B viral genome:

1. the 5' oligonucleotide having 14 nucleotides (5'-TACGGACAGAAACT-3') covers positions 582 to 595. Specifically, it contains the mutation G to A at position 8 of the said oligonucleotide, leading to change at amino acid 145 (Glycine to Arginine) of the human hepatitis B virus surface antigen;

2. the 3' oligonucleotide having 21 nucleotides (5'-TTAGGGTTTAAATCTATACCC-3') covers positions 842 to 822. Specifically, it is an anti-sense oligonucleotide that is complementary to the coding strand of human hepatitis B virus surface antigen.

Cycling conditions of polymerase chain reaction using the above-mentioned oligonucleotides are as follows: 1.5 minutes at denaturing temperature (94 °C), 2 minutes at annealing temperature (50 °C) and 2 minutes at extension temperature (72 °C). Amplified product is visualized after eletrophoresis on an 2% agarose gel. A total of 35 cycles using *Pfu* polymerase (Stratagene, U.S.A.) generate an amplified product of expected size (240 base pairs) for plasmid (*lane 2* in Figure 1) and viral DNA (*lane 4* in Figure 1) carrying human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine). The specificity of the detection system in the present invention is indicated by the absence of amplified product using templates carrying the wild type human hepatitis B virus surface antigen (plasmid template in *lane 1*, and viral DNA in *lane 3* of the Figure 1). The said specificity is further supported by the absence of amplified product using templates carrying mutations leading to amino acid changes at position 126, 129 and 133 of the human hepatitis B virus surface antigen.

EXAMPLE 1

Detection of human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) on solid glass supports (Gene Chip)

An increasing number of human hepatitis B virus mutants are being identified. Whereas some of them derive from random variations during viral replication cycles, many

others emerge from selection pressure such as immunoprophylaxis with vaccines and therapeutic treatment with antiviral drugs. These 'escape' mutants are of concern as they are generally replicative and some can be infectious leading to acute liver diseases. Identification of such human hepatitis B virus mutants from serum samples is therefore of great importance. One of the most powerful approaches would be a differential amplification by mutation-specific oligonucleotide probes, as described in the present invention. With appropriate selection of oligonucleotide and amplification conditions, our novel detection system in the present invention allows the discrimination between a target human hepatitis B virus and the said mutant 145 (Glycine to Arginine) by only a single base. However, the manual and laborious gel-based analysis of amplified products could comprise its use in routine detection of the increasing numbers of human hepatitis B virus mutants, in particular those carrying mutations on the antigenic 'a' epitope of the viral surface antigen.

A promising alternative approach to this problem would be the development of analytical device that allows the simultaneous detection of different mutations, such as an array of hundreds or thousands of immobilized oligonucleotides (gene chip). In the case of human hepatitis B virus surface antigen, solid glass supports with immobilized oligonucleotides that carry specific mutations would allow their simple and rapid detection.

As a direct application of the novel detection system in the present invention, modifications have been added to two oligonucleotides (listed in Claim 5): 5'-TACGGACGGAAACT-3', and 5'-TACGGACAGAAACT-3', both located from position 582 to 595 as referred to the wild type human hepatitis B virus genome. These include a fluorescent dye, 6-(fluorescein-6-carboxamido)

hexanoate, at its 5′ terminus for microscopic detection and a primary amine group at its 3′ terminus allowing its immobilization on solid glass supports. The resulting oligonucleotides that are immobilized on solid glass supports has the following structure: 5′-(6FAM)TACGGACGGAAACTGTTTTTTTTTTTT (C-7 amine)-3′, and 5′-(6FAM)TACGGACAGAAACTGTTTTTTTTTTTT (C-7 amine)-3′, and the second oligonucleotide contains the mutation G to A (position 8 of the oligonucleotide, *in bold*) leading to change at amino acid 145 (Glycine to Arginine) of human hepatitis B virus surface antigen. There is also an inclusion of a poly-T (underlined) as a synthetic linker aiming at optimizing the subsequent hybridization reaction with target human viral DNA sequences from serum samples.

Activation of Glass Slides

The said modified oligonucleotides are then immobilized on solid glass supports that are activated as follows:

- immerse glass slides in 1% 3-aminopropyltrimethoxysilane solution in 95% acetone/water for 2 minutes;
- wash glass slides in acetone;
- dry glass slides at 100 °C for 45 minutes;
- incubate glass slides in 0.2% 1-4-phenylene diisothiocyanate in 10% pyridine/dimethyl formamide for 2 hours;
- wash glass slides with methanol and acetone;
- store glass slides in vacuum desiccator.

Immobilization of Oligonucleotides

- the oligonucleotides:
  1. oligonucleotide having 27 nucleotides with a fluorescent dye, 6-(fluorescein-6-carboxamido) hexanoate (6FAM), at its 5′ terminus and a primary amine group at its 3′ terminus: 5′-(6FAM)TACGGACGGAAACTGTTTTTTTTTTTT (C-7

amine)-3'; and

2. oligonucleotide having 27 nucleotides with a fluorescent dye, 6-(fluorescein-6-carboxamido) hexanoate (6FAM), at its 5' terminus and a primary amine group at its 3' terminus: 5'-(6FAM)TACGGACAGAAACTGTTTTTTTTTTTT (C-7 amine)-3'

are dissolved in 100 mM Na_2CO_3 (pH 9.0) to a final concentration of 20 nM;

- apply 2 μl of the above solution containing oligonucleotides to the activated glass slides;
- incubate glass slides with immobilized oligonucleotides at 37 °C for 2 hours in a humid chamber;
- wash glass slides in 1% NH_4OH and water, allow air dry at ambient temperature.

Amplification of Target Viral DNA

Specific oligonucleotides that can be used to amplify target human hepatitis B virus surface antigen DNA from serum samples are designed, prior to their hybridization with the oligonucleotide immobilized on solid glass supports as mentioned above. Specifically, these oligonucleotides consist of the following structure:

- first oligonucleotide having 20 nucleotides with a biotin group at its 5' terminus (5'-Biotin-AGGATCAACAACAACCAGTA-3', and located from position 489 to 508 as referred to the wild type human hepatitis B virus genome). The presence of a biotin group allows the separation of amplified DNA fragments using streptavidin magnetic particles;
- second oligonucleotide having 20 nucleotides with a fluorescent dye Texas Red at its 5' terminus, and complementary to the coding strand of human hepatitis B virus surface antigen (5'-Texas

red-ATCGTCCTGGGCTTTCGCAA-3'), and is located from position 634 to 615 as referred to the wild type human hepatitis B virus genome;

- polymerase chain reaction is carried out using viral DNA (either wild type or mutant 145 with Glycine to Arginine mutation of human hepatitis B virus surface antigen) from serum samples as template, and generates an amplified product of 150 base pairs;
- separate double stranded amplification product by immobilizing the biotinylated coding strand onto streptavidin magnetic particles;
- solution containing single-stranded DNA with Texas Red fluorescent label is collected for subsequent hybridization with immobilized oligonucleotides.

Hybridization and Detection of Fluorescent Signals

- apply hybridization solution containing 50 nM Texas Red-labeled target single stranded DNA, 5X SSPE (NaCl, NaH_2PO_4, and EDTA) and 0.5% SDS (Sodium Dodecyl Sulfate) to glass slides carrying immobilized oligonucleotides at 30 °C for 3 hours;
- wash with 2X SSPE and 0.1% SDS;
- apply 50 μl of the above washing buffer to solid glass supports, cover with cover slips and detect fluorescence signals under fluorescent microscope.

Detection of fluorescence signals under microscope indicates a specific hybridization between the immobilized oligonucleotide: 5'-(6FAM)TACGGACAGAAACTGTTTTTTTTTTTT (C-7 amine)-3', with the G to A mutation (leading to Glycine to Arginine mutation at amino acid 145 of human hepatitis B virus surface antigen) and the target viral DNA carrying the same mutation. Conversely, specific hybridization occurs exclusively between the immobilized wild type oligonucleotide, 5'-(6FAM)TACGGACGGAAACTGTTTTTTTTTTTT

(C-7 amine)-3',and the target viral DNA amplified from wild type human hepatitis B virus.

The present invention is based on a simple and specific system to detect human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) from serum samples. This specific detection provides useful information for further monitoring of this particular mutant. The successful application of this detection system onto solid glass supports makes fast and specific detection of the said human hepatitis B viral mutant at large scale possible. The detection system in the present invention can also be extended to other human hepatitis B viral mutants, including those emerged from selection pressure (either vaccine-based or therapeutic drug-based).

Further variations and modifications of the present invention will become apparent subsequently and are to be covered by the claims included in the present invention.

Other Publications

Carman, W.F., et al. "Hepatitis B virus envelope variation after transplantation with and without hepatitis B immune globulin prophylaxis", Hepatology (1996), vol. 24, pp. 489-493.

Guo. Z., et al. "Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports", Nucl. Acids Res. (1994), vol. 22, pp. 5456-5465.

Hoheisel J. Oligomer-chip technology. Trends Biotechnol. (1997), vol. 15, pp. 465-469.

Goffeau, A., "Molecular fish on chips", Nature (1997), vol. 385, pp. 202-203.

Hsu, H.Y., et al. "Surface gene mutants of hepatitis B virus in infants who develop acute or chronic infections despite immunoprophylaxis", Hepatology (1997), vol. 26, pp. 786-791.

Marshall A. and Hodgson J. "DNA chips: an array of possibilities." Nat. Biotechnol. (1998), vol. 16, pp. 27-31.

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Oon, C.J., et al. "Molecular epidemiology of hepatitis B virus vaccine variants in Singapore", Vaccine (1995), vol. 13, pp. 699-702.

Saiki R.K., et. al. "Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes." Proc. Natl. Acad. USA (1989), vol. 86, pp. 6230-6234.

Yershov G., Barsky V. et. al. "DNA analysis and diagnostics on oligonucleotide microschips." Proc. Natl. Acad. Sci. USA (1996), vol. 93, pp. 4913-4918.

What is claimed is:

1. A set of synthetic oligonucleotides consisting of a 5' and 3' oligonucleotide for polymerase chain reaction of hepatitis B virus, wherein the 5' oligonucleotide is a 14mer which contains a mutation leading to amino acid changes in human hepatitis B virus proteins leading to escape viral mutants, including those derived from immunoprophylaxis with vaccines and hepatitis B immune globulin and from treatment with antiviral drugs and the 3' oligonucleotide with an appropriate size which is conserved between the mutant and wild type strains.

2. The oligonucleotides of claim 1, wherein the 5' oligonucleotide has the sequence, 5'-TACGGACAGAAACT-3', which corresponds to position 582 to 595 in the wild type human hepatitis B virus genome and contains the mutation G to A, leading to change at amino acid 145 of hepatitis B virus surface antigen from Glycine to Arginine, at position 8 of the said oligonucleotide.

3. The oligonucleotide of claim 1, wherein the 3'oligonucleotide has sequence, 5'-TTAGGGTTTAAATCTATACCC-3', which corresponds to position 842 to 822 in the wild type human hepatitis B virus genome and is complementary to the coding strand of human hepatitis B virus surface antigen.

4. A composition comprising an amount of the oligonucleotides of the claim 1, 2 or 3 suitable for polymerase chain reaction.

5. A method of determining the presence of human

hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) in a sample comprising:

a) obtaining the sample;

b) treating the obtained sample for uses in a polymerase chain reaction; and

c) amplifying the treated sample with the two oligonucleotides in claim 1, 2 or 3, wherein if the sample is amplified will indicate that the sample contains human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine).

6. The method of claim 5, wherein the sample is a serum sample.

7. An oligonucleotide of appropriate size, containing a mutation leading to amino acid changes in human hepatitis B virus proteins leading to escape viral mutants, including those derived from immunoprophylaxis with vaccines and hepatitis B immune globulin and from treatment with antiviral drugs, wherein the 5' terminus of the oligonucleotide is with a fluorescent dye, 6-(fluorescein-6-carboxamido) hexanoate (6FAM); the 3' terminus is a primary amine group; and a poly-T linker consisting of 12 T is preceding the 3' primary amine group.

8. The oligonucleotide of claim 7, wherein the length of the oligonucleotide corresponding to the viral genome is 14 to 20 nucleotides.

9. A set of oligonucleotides useful for immobilizing on solid supports, wherein each pair of oligonucleotides consist of:

A. a first oligonucleotide having 27 nucleotides with a first fluorescent dye, at its

5'terminus and a primary amine group at its 3' terminus: 5'-(6FAM)TACGGACGGAAACTGTTTTTTTTTTTT (C-7 amine)-3'; wherein the first fifteen nucleotides correspond to wild type human hepatitis B virus genome position 580 to 594, and the poly-T is a synthetic linker aiming at facilitating the subsequent hybridization reaction with target viral DNA sequences from serum samples; and

B. second oligonucleotide having 27 nucleotides with a second fluorescent dye at its 5' terminus and a primary amine group at its 3' terminus with a sequence, 5'-dye-TACGGACAGAAACTGTTTTTTTTTTTTC-7 amine-3'; wherein the first fifteen mucleotides contain the mutation G to A at position 8 of the said second oligonucleotide, *in bold*, leading to change at amino acid 145 (Glycine to Arginine) of human hepatitis B virus surface antigen, and correspond to wild type human hepatitis B virus genome position 580 to 594, and wherein the poly-T is a synthetic linker aiming at facilitating the subsequent hybridization reaction with target human viral DNA sequences from serum samples.

10. The synthetic oligonucleotides of claim 9, wherein the first and the second fluorescent dye is the same.

11. The synthetic oligonucleotides of claim 10, wherein the dye is 6-(fluorescein-6-carboxamido) hexanoate (6FAM).

12. A method to screen for human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine), wherein the specific oligonucleotides in claim 9

with chemical modifications: a fluorescent dye at 5' terminus and primary amine group at 3' terminus, are immobilized on solid supports.

13. A method to screen for human hepatitis B virus mutants, derived either from immunoprophylaxis with hepatitis B immune globulin and vaccines or from treatment with antiviral drugs, or from asymptomatic hepatitis B virus carriers, wherein the specific oligonucleotides in claim 9 with chemical modifications: a fluorescent dye at 5' terminus and primary amine group at 3' terminus, are immobilized on solid supports.

14. The method of claim 12 or 13, wherein the solid supports are glasses.

15. A set of oligonucleotides useful as amplifier probes in a polymerase chain reaction for human hepatitis B viral DNA, wherein the size of generated amplification product should be under 150 base pairs; wherein the generated amplification product should cover the said mutation site and in particular the G to A mutation leading to amino acid Glycine to Arginine change at position 145 of human hepatitis B virus surface antigen; wherein said oligonucleotides comprise at least two different oligonucleotides probes which are:

A. first oligonucleotide having 20 nucleotides with a biotin group at its 5' terminus, 5'-Biotin-AGGATCAACAACAACCAGTA, position 489 to 508 as referred to the wild type human hepatitis B virus genome, wherein the presence of a biotin group allows the separation of amplified DNA fragments using streptavidin magnetic particles; and

B. second oligonucleotide having 20 nucleotides with a fluorescent dye Texas Red at its 5' terminus, and complementary to the coding strand of human hepatitis B virus surface antigen with the sequence, 5'-Texas red-ATCGTCCTGGGCTTTCGCAA-3', position 634 to 615 as referred to the wild type human hepatitis B virus genome.

16. A method to amplify target DNA of human hepatitis B virus surface antigen from serum samples, by polymerase chain reaction using the oligonucleotides in claim 15 that contain modifications: addition of biotin and Texas red groups for sense and anti-sense oligonucleotides respectively, at their 5' terminus.

17. A set of oligonucleotides useful as amplifier probes in a polymerase chain reaction for human hepatitis B viral DNA, wherein the size of generated amplification product should be under 150 base pairs; wherein the generated amplification product should cover mutations that result in amino acid changes in human hepatitis B virus proteins leading to escape viral mutants, including those derived from immunoprophylaxis with vaccines and hepatitis B immune globulin and from treatment with antiviral drugs; wherein said oligonucleotides comprise at least two different oligonucleotides probes which are:

A. a first oligonucleotide having 20 nucleotides with a biotin group at its 5' terminus, wherein the presence of a biotin group allows the separation of amplified DNA fragments using streptavidin magnetic particles; and

B. a second oligonucleotide having 20 nucleotides

with a fluorescent dye Texas Red at its 5' terminus, and located within 150 base pairs downstream of the first oligonucleotide on human hepatitis B virus genome.

18. A method to amplify target DNA of human hepatitis B virus that may carry mutations following either immunoprophylaxis with hepatitis B immune globulin and vaccines or treatment with antiviral drugs. The said amplification is by polymerase chain reaction using the oligonucleotides in claim 17 that contain modifications: addition of biotin and Texas red groups for sense and anti-sense oligonucleotides respectively, at their 5' terminus.

DETECTION OF HUMAN HEPATITIS B VIRUS SURFACE ANTIGEN MUTANTS BY SPECIFIC AMPLIFICATION AND ITS APPLICATION ON GENE CHIP

Abstract of the Invention

Novel DNA probe sequences for detection, by polymerase chain reaction, of human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) from serum samples. As a direct application, these specific DNA probes are immobilized on solid glass supports (gene chip) for detection of human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine) by fluorescence.

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Figure 1

Figure 1. Detection of human hepatitis B virus surface antigen mutant 145 (Glycine to Arginine)